PTO 03-5358

French Patent

2,596,064

# Industrial Methods for Manufacturing Ribosomal Vaccines and Obtained Ribosomal Vaccines

[Procédés industriels de fabrication de vaccins ribosomaux et vaccins ribosomaux obtenus]

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UNITED STATES PATENT AND TRADEMARK OFFICE

Washington, D.C. September 2003

Translated by: Schreiber Translations, Inc.

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| Country | : | France |
| Document No. | : | 2,596,064 |
| Document Type | : | Patent |
| Language | : | French |
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| IPC | : | C 12 P 21/00, 1/04; A 61 K 39/02, 39/116; C 07 K 15/04, 15/14//(C 12 P 21/00, C 12 R 1:22) (C 12 R 1/15, 1:425). |
| Application Date | : | March 18th, 1986 |
| Publication Date | : | September 25th, 1987 |
| Foreign Language Title | : | Procédés industriels de fabrication de vaccins ribosomaux et vaccins ribosomaux obtenus |
| English Title | : | Industrial Methods for Manufacturing Ribosomal Vaccines and Obtained Ribosomal Vaccines |

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The present invention relates to industrial methods for manufacturing ribosomal vaccines and the ribosomal vaccines derived therefrom.

Ribosomal vaccines are vaccines wherein the antigenic fraction is composed of a ribosomal fraction; this antigenic ribosomal fraction must, in general, be adjuvated by a fraction that may also be of microbial origin. In most cases, this involves glycoproteins of membrane origin and/or polysaccharides or lipopolysaccharides of microbial origin.

The applicant's prior patent, No. 7343957 dated December 10th, 1973, described the preparation of antigenic ribosomal fraction-based vaccines adjuvated by a membrane fraction of Klebsiella pneumoniae.

The applicant's prior patents No. 7510252 dated April 2nd, 1975 and No. 7624124 dated August 6th, 1976 described vaccines derived from ribosomal vaccines wherein the ribosomal RNA or the ribosomes themselves were associated with homologous and/or heterologous polysaccharide or lipopolysaccharide fractions.

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[1] Numbers in the margin indicate pagination in the foreign text.

The applicant's prior patent No. 7835649 dated December 19th, 1978 described the preparation of bacterial membrane proteoglycans that are usable as adjuvants in ribosomal vaccines.

The present invention, developed at the Centre d'Immunologie et de Biologie Pierre Fabre, relates to novel industrial methods for the manufacture of immunogenic microbial ribosomal fractions as well as membrane proteoglycans of nonencapsulated biotype-a Klebsielle pneumoniae that are associated with them as adjuvants.

This novel technology enables the development of very large-scale production of this type of vaccines, particularly for veterinary applications such as those cited by way of example.

More specifically, the present invention relates to a method for preparing membrane proteoglycans from bacteria selected from among: Klebsiella, Serratia, and Corynebacterium, wherein, starting from a clarified lysate of said bacterial strain:

a) the supernatant of the clarified lysate is processed with CTAB or trichloroacetic acid, and

b) after processing, the supernatant containing the purified membrane proteoglycans is separated from the precipitated impurities.

Generally speaking, bacterial lysates are obtained by milling a bacterial concentrate using mechanical and/or pneumatic means; these milling means must, of course, be adapted to the type of microbial cells to be disintegrated and more specific information will be provided in the description of the examples.

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The bacterial lysates obtained after milling may be clarified using any known technology, but are preferably clarified by centrifugation at 15,000 g, for example, on a separator; this is to eliminate milling residue and unmilled germs.

Depending upon the type of processing performed during step a), we proceed as follows:

- When processing with CTAB is performed, the supernatant is separated by centrifugation and ultracentrifugation is performed in order to eliminate the CTAB itself, salts, and low-molecular-weight contaminants. This is done by using, for example, tangential ultrafiltration with a membrane cutting at 10,000 d;

- when the processing involves processing with trichloroacetic acid, the precipitate of impurities is eliminated by centrifugation, on a separator for example, and the supernatant is ultrafiltered on a membrane cutting at 10,000 d as above, but preferably after bringing the pH up to around 7, to 7.8 for example, by adding a base such as concentrated NaOH.

In both cases, after ultrafiltration, we obtain a concentrated suspension of membrane proteoglycans, which may be sterilized by autoclaving at 120°C, then lyophilized in order to be used at a later date.

Preferably, the adjuvant membrane proteoglycans will be prepared from a nonencapsulated biotype-a mutant strain of Klebsiella pneumoniae, recorded in the Collection of the Institut Pasteur in Paris under the number 145-I.IP.

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The antigenic ribosomal fraction is preferably prepared as follows:

a) a filtered and clarified lysate of this strain is processed with $MgCl_2$ using an organic acid or CTAB;

b) after processing, the precipitate is recovered; it may be washed in order to recover the ribosomes, if necessary.

The filtered, clarified lysate may be obtained for the clarified lysate as described above for the membrane

proteoglycans; however, the clarified lysate is filtered, preferably using ultrafiltration on a membrane cutting at 0.22 μ in order to eliminate, specifically, the membrane proteoglycans and the various milling residues that may not have been eliminated during centrifugation.

Depending upon the nature of the processing used in step a), we proceed as follows:

- When the processing is performed with $MgCl_2$, we add to the clarified and filtered bacterial lysate a solution of $MgCl_2$, preferably 1 N, until we obtain a final concentration of 0.1 M of $MgCl_2$. The pH is adjusted to an acid pH, on the order of 6 for example, using diluted HCl, and the ribosomes are allowed to precipitate out. This precipitate is then collected using centrifugation, for example.

- When the processing is performed using an organic acid, specifically acetic acid, the acetic acid is added until a pH of 4 is reached, then the ribosome precipitate is allowed to form and it is separated by centrifugation, for example.

- When the processing involves CTAB, we add to the clarified and filtered bacterial lysate a CTAB solution, at 5% for example (w/v), and the precipitate is allowed to form. This precipitate is recovered by centrifugation and may be washed using ethanol, for example.

The product obtained at the end of step b) constitutes an unwashed ribosomal fraction that can be purified by washing with sodium dodecylsulfate, then by precipitation using ethyl alcohol, for example. The obtained precipitate can be subjected to additional purification by immunoaffinity column chromatography of the immobilized monoclonal antibodies that are specific to the vaccinating antigenic determinant linked to the ribosomes. The highly-purified ribosomes obtained in this way may be dialyzed in order to eliminate excess salts, then sterilized before being used in association with the membrane proteoglycans.

The nature of the strains that are processed to obtain the ribosomes depends, of course, on the type of vaccines one wishes to obtain, since these ribosomes constitute the antigenic fraction of the vaccines.

We will provide hereinafter various vaccine formulas that belong more specifically within the scope of the invention. In general, the strains used originate either from pathology samples or microorganism collections; these strains were reactivated in laboratory animals and maintained on appropriate media.

Among the vaccines that may be obtained using the method according to the invention, we must cite more specifically:

1) Parodontopathy vaccine

- Single-dose formula

| | | |
|---|---|---|
| a) | - Ribosomes of Actinomyces viscosus | 3 µg |
| | - Ribosomes of Actinomyces naeslundii | 2 µg |
| | - Ribosomes of Veillonella parvula | 2 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 15 µg |

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2) Intestinal vaccine

- Single-dose formula

| | | |
|---|---|---|
| b) | - Ribosomes of Escherichia coli | 3 µg |
| | - Ribosomes of Salmonella typhimurium | 3 µg |
| | - Ribosomes of Shigella dysenteriae | 3 µg |
| | - Ribosomes of Staphylococcus aureus | 3 µg |
| | - Membrane proteoglycans of Kelbsiella pneumoniae | 18 µg |

3) Candidoses vaccine

- Single-dose formula

| | | |
|---|---|---|
| c) | - Ribosomes of type A Candida albicans | 3.5 µg |
| | - Ribosomes of type B Candida albicans | 3.5 µg |

- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

4) Meningitis vaccine

- Single-dose formula

d) - Ribosomes of group A Neisseria meningitidis 3.5 µg
- Ribosomes of group C Neisseria meningitidis 3.5 µg
- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

- Single-dose formula

e) - Ribosomes of type a Haemophilus influenzae 3.5 µg
- Ribosomes of type b Haemophilus influenzae 3.5 µg
- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

5) Pneumococcal vaccine

- Single-dose formula

f) - Ribosomes of type 1 Streptococcus pneumoniae 3 µg
- Ribosomes of type 2 Streptococcus pneumoniae 3 µg
- Ribosomes of type 4 Streptococcus pneumoniae 3 µg
- Ribosomes of type 19 Streptococcus pneumoniae 3 µg
- Membrane proteoglycans of Klebsiella pneumoniae 20 µg.

6) Trivalent streptococcal porcine meningitis vaccine

- Single-dose formula

g) - Ribosomes of group L Streptococcus pyogenes 5 µg

- Ribosomes of group C Streptococcus pyogenes 5 µg

- Ribosomes of Streptococcus suis 5 µg

- Membrane proteoglycans of biotype a Klebsiella pneumoniae 10 µg

7) Rabbit staphylococcia vaccine

- Trivalent vaccine single-dose formula

h) - Ribosomes of Staphylococcus epidermidis 1.5 µg

- Ribosomes of type 7 Staphylococcus aureus 1.5 µg

- Ribosomes of Staphylococcus aureus 1.5 µg

- Membrane proteoglycans of biotype a Klebsiella pneumoniae 7.5 µg

- Divalent vaccine single-dose formula

i) - Ribosomes of Staphylococcus (Mérimée strain) 3.5 µg

- Ribosomes of Staphylococcus aureus 3.5 µg

- Membrane proteoglycans of biotype a Klebsiella pneumoniae 15.0 µg

8) Cow mastitis vaccine

- Single-dose formula

j) - Ribosomes of Staphylococcus aureus 5 µg

- Ribosomes of Staphylococcus uberis 5 µg

- Ribosomes of Streptococcus dysgalactiae 5 µg

- Membrane proteoglycans of biotype a Klebsiella pneumoniae 20 µg

These vaccines are obtained by mixing previously-obtained membrane proteoglycans with ribosomes obtained using the described methods; the ratios by weight of the ribosomes to the membrane proteoglycans preferably range from 1/1 to 1/3 counted for the entirety of the ribosomal fraction in relation to the membrane proteoglycan fraction.

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The following examples are more specifically intended to emphasize other characteristics and advantages of the invention.

EXAMPLE 1 - OBTAINING CLARIFIED BACTERIAL LYSATES

This example describes the preferred method for preparing clarified bacterial lysates, whether they are intended for use in preparing membrane proteoglycans or in preparing ribosomal fractions; this explains why no specific mention is made of microorganism strains.

The microbial biomasses are obtained by growing them in a liquid-medium fermenter under traditional conditions. The only specific constraint was that growth was abruptly stopped by

cooling to +4°C at the end of the exponential growth phase in order to preserve the integrity of the biological structures.

The biomass is separated from the culture medium by continuous cold centrifugation on commercial separators of the Sharples or Westfalia type.

After washing by replacing them in suspension in sterile physiological serum and centrifugation, the cell concentrates are stored frozen until they are processed.

The bacterial concentrate is thawed in a reactor and suspended in tris-HCl buffer (10 mM), pH 7.0, containing $MgCl_2$ (0.15 M) at 4°C, resulting in a final concentration of 50 g of dry cells per liter of suspension. We then add 5 mg of RNase-free DNase per liter of suspension.

The microbial cells are then disintegrated by continually passing them through commercial mills of the three-stage APV Manton Gaulin type for low-resistance strains or through a glass bead homogenizer of the DYNO MILL type for highly-resistant cocci and strains. This operation is performed at low temperature, ≤ 4°C, using a high-power heat exchanger.

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The previously-obtained bacterial lysates are subjected to an initial continuous clarification at 15,000 g on a Sharples

separator at +4°C in order to eliminate milling residues and unmilled germs.

The centrifugation pellet is eliminated and the supernatant is collected: it makes up the clarified lysate.

OBTAINING MEMBRANE PROTEOGLYCANS OF KLEBSIELLA PNEUMONIAE (PGM-Kp) - RIBOSOME ADJUVANTS

The methods enabling very large-scale production of (PGM-Kp) are described below starting with the Sharples-clarified Klebsiella pneumoniae bacterial lysate obtained in Example 1.

EXAMPLE 2

50 l of Sharples supernatant (clarified bacterial lysate) from Example 1 obtained from Klebsiella pneumoniae No. 145 are placed in a reactor, stirring at 4°C. We progressively add to this suspension 1 volume of a 1% solution (w/v) of cetyl trimethyl ammonium bromide (CTAB), then it is allowed to sit for a few minutes.

The precipitate of impurities (nucleic acids, proteins, acid polysaccharides, etc.) is eliminated by continuous centrifugation on a Sharples separator and the supernatants containing the (PGM-Kp) are collected.

The (PGM-Kp) are then purified in a tangential ultrafiltration step using a Millipore apparatus equipped with membranes cutting at 10,000 d. This ultrafiltration, dialysis,

and concentration step enables the quantitative elimination of CTAB, salts, and low-molecular-weight contaminants.

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The concentrated suspension of (PGM-Kp) thus obtained is sterilized by autoclaving for 20 minutes at 120°C, then sterilely lyophilized.

EXAMPLE 3

50 l of Sharples supernatant (clarified bacterial lysate from Example 1 obtained from Klebsiella pneumoniae No. 145) are placed in a reactor, stirring at +4°C. We add to the reactor 2500 g of crystallized trichloroacetic acid, then stir until it completely dissolves.

After allowing it to sit for 20 minutes at 4°C, the precipitate of impurities is eliminated by continuous centrifugation on a Sharples separator and the supernatant containing the (PGM-Kp) is collected.

The solution's pH is then brought to 7.8 pH by adding concentrated NaOH, then we proceed with tangential ultrafiltration on a membrane cutting at 10,000 d as in Example 2.

The concentrated suspension of (PGM-Kp) thus obtained is sterilized by autoclaving for 20 minutes at 120°C, then sterilely lyophilized.

OBTAINING THE UNWASHED RIBOSOMAL FRACTION

All of these methods follow the common principle of causing a specific precipitation of the ribosomes that allows them to be separated by low-speed continuous centrifugation, thus obviating the need for very high-speed ultracentrifugation, which only applies to small volumes.

The following examples were implemented starting with bacterial lysates obtained as described in Example 1 from the appropriate bacterial strain; the collected supernatant undergoes tangential ultrafiltration on a Millipore system equipped with membranes cutting at 0.22 μ.

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The clear filtrate obtained then contains the ribosomes and the soluble substances that will be separated as described below.

EXAMPLE 4

50 l of ultrafiltered bacterial lysate are placed in a reactor at +4°C and a solution of 1 M of $MgCl_2$ is slowly added until a final concentration of 0.1 M of $MgCl_2$ is obtained. The pH is then brought to 6.0 using diluted HCl and the ribosomes are left to precipitate out for one hour at 4°C while being stirred very slowly.

The precipitate is collected by continuous centrifugation on Sharples then placed back into a solution in an initial volume of Tris-HCl buffer (10 mM), pH 7.0, containing EDTA $Na_2$ (5 mM).

This solution constitutes the unwashed ribosomal fraction.

EXAMPLE 5

50 l of ultrafiltered bacterial lysate are placed in a reactor at +4°C and the pH of the suspension is progressively lowered until it reaches pH 4.0 by adding acetic acid (roughly 0.5% of the volume).

After resting for 1 hour at +4°C, the precipitate of unwashed ribosomes is separated by continuous centrifugation on Sharples separator.

The pellet is energetically dispersed in an initial volume of Tris-HCl buffer (10 mM), pH 7.5, containing NaCl (0.15 M) and EDTA $Na_2$ (1 mM) while maintaining a constant pH.

This solution constitutes the unwashed ribosomal fraction.

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EXAMPLE 6

50 l of ultrafiltered bacterial lysate are placed in a reactor at +4°C while stirring. 5 l of a 5% solution (w/v) of CTAB (cetyl trimethyl ammonium bromide) is slowly added.

After resting for several minutes, the precipitate is collected by continuous centrifugation on Sharples.

The precipitate is washed twice by dispersion [for] 30 minutes in 30 l of 70% ethanol, 0.2 M of $CH_3$ CO Ona, pH 7.0, then centrifugation on Sharples.

The washed pellet is placed back into 30 l of Tris-HCl buffer (10 mM), pH 7.0, containing $MgCl_2$ (10 mM) and NaCl (0.15 M).

This solution constitutes the unwashed ribosomal fraction.

EXAMPLE 7 - OBTAINING PURIFIED RIBOSOMES

The unwashed ribosomal suspension obtained by one of the three methods described above undergoes purification including the following steps:

1) Washing of the ribosomes

The impurities connected to the ribosomes are eliminated by washing with SDS (sodium dodecyl sulfate) under the following conditions: 30 l of a solution of unwashed ribosomes are placed in a reactor while stirring and the temperature is brought to 20°C. To this solution is added 75 g of SDS, then stirring is continued for 45 minutes at this temperature; the medium quickly becomes clear.

2) Precipitation of the purified ribosomes

To the preceding solution, we quickly add while stirring 21 l of iced ethyl alcohol, then it is allowed to sit for 30 minutes at 4°C.

The precipitate of purified ribosomes is collected using continuous centrifugation on Sharples separator and the pellet is placed in 10 l of iced pH 7.0 buffer containing $MgCl_2$ (1 mM) and KCl (0.05 M).

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3) Purification by affinity chromatography

Monoclonal antibodies are prepared against each ribosome by hyperimmunizing the mouse with these ribosomes and by fusing immune spleen cells from the mouse with x 63 myeloma cells using the technique described by G. Galfre and C. Milstein (Methods in Enzymol. 73 B, pp. 3-46 (1981)). The monoclonal antibodies thus obtained are selected based on their affinity with the ribosomes and their ability to induce passive protection in the mouse against a homologous infection.

A specific affinity support is then prepared by covalent coupling of this monoclonal antibody on a dextran-type matrix activated by a traditional method such as CNBt, for example.

An affinity column is thus prepared that enables selective isolation of the immunogenic ribosomes according to the protocol described below:

- a column 70 mm in diameter and 400 mm high is molded with the affinity support prepared as described above and balanced in 10 mM Tris-HCl buffer, pH 7.0, containing $MgCl_2$ (1 mM) and KCl (0.05 M).

- The previously-prepared solution of washed ribosomes is passed onto the column in order to set the ribosomes. The column is then washed by the same buffer until the effluent no longer absorbs at 280 nm.

- The ribosomes are then eluted by the same buffer also containing KSCN (2 M). The ribosome peak detected by its absorption at 260 nm is collected.

4) Ultrafiltration

The purified ribosomal fraction undergoes ultrafiltration on membrane cutting at 100,000 d with supply of iced, 7.0 pH buffer containing $MgCl_2$ (1 mM) and KCl (0.05 M) in order to fully eliminate the KSCN and to concentrate the solution in order to yield roughly 10 mg/ml of ribosomes.

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EXAMPLE 8 - PREPARATION OF THE RIBOSOMAL VACCINE

To make the ribosomal vaccine in its definitive form, the following operations are performed in a closed, sterile area:

- thawing of the ribosomal suspensions,

- grouping [of the suspensions] in the proportions of the formula under consideration depending upon their respective ribosome strengths,

- adding the corresponding quantity of lyophilized membrane proteoglycans of Klebsiella pneumoniae,

- homogenizing,

- adding excipients according to the final form being considered,

- distribution into single-dose containers (or by multiples of the single dose amount),

- sterile lyophilization,

- capping, crimping,

- testing (physico-chemical, immunological, sterility, toxicity, etc.).

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CLAIMS

1) Method for preparing membrane proteoglycans from bacteria selected from among: Klebsiella, Serratia, and Corynebacerium, wherein, starting with a clarified lysate of the bacterial strain,

a) the clarified lysate supernatant is processed with cetyl trimethyl ammonium or trichloroacetic acid, and

b) after processing, the supernatant containing the purified membrane proteoglycans is separated from the precipitated impurities.

2) Method according to Claim 1, wherein the lysate is obtained by milling a bacterial concentrate using mechanical and/or pneumatic means.

3) Method according to either of claims 1 or 2, wherein the lysate is clarified by centrifugation.

4) Method according to one of claims 1 through 3, wherein, after processing with cetyl trimethyl ammonium, the supernatant is separated by centrifugation and ultrafiltration is performed in order to eliminate the cetyl trimethyl ammonium, salts, and low-molecular-weight contaminants.

5) Method according to one of claims 1 through 3, wherein, after processing with trichloroacetic acid, the supernatant is separated by centrifugation and ultrafiltration is performed, after bringing the pH to between 7 and 8, in order to eliminate the salts and low-molecular-weight contaminants.

6) Method according to one of claims 4 or 5, wherein the ultrafiltration is a tangential ultrafiltration on a membrane cutting at 10,000 daltons.

7) Method according to one of claims 1 through 6, wherein the bacterial strain is a strain of unencapsulated biotype a Klebsiella pneumoniae.

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8) Membrane proteoglycans obtained by implementing the method according to one of claims 1 through 5.

9) Method for preparing ribosomal fractions from bacterial strains, wherein:

a) a filtered, clarified lysate of the strain is processed using $MgCl_2$, an organic acid, or cetyl trimethyl ammonium,

b) after processing, the precipitate is recovered; it may be washed in order to recover the ribosomes.

10) Method according to Claim 9, wherein, in step a), processing is performed until a concentration of 0.05 to 0.2 M of $MgCl_2$ at an acid pH is obtained and wherein the precipitated ribosomes are collected.

11) Method according to Claim 9, wherein, in step a), processing is performed by lowering the pH to roughly 4 using acetic acid and wherein the precipitated ribosomes are collected.

12) Method according to Claim 9, wherein, in step a), processing is performed using cetyl trimethyl ammonium and wherein, in step b), the precipitate is washed using ethanol.

13) Method according to one of claims 9 through 12, wherein the ribosomes obtained in step b) are washed using sodium dodecylsulfate, then precipitated using ethanol.

14) Method according to one of claims 9 through 13, wherein the clarified lysate is filtered by ultrafiltration on membrane cutting at 0.22 µm.

15) Ribosomes obtained by the implementation of the method according to one of claims 9 through 14.

16) Ribosomal vaccines wherein they include, in association, ribosomes according to Claim 15 and membrane proteoglycans according to Claim 8.

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17) Vaccines according to Claim 16, wherein each single-dose amount contains:

| | | |
|---|---|---|
| a) | - Ribosomes of Actinomyces viscosus | 3 µg |
| | - Ribosomes of Actinomyces naeslundii | 2 µg |
| | - Ribosomes of Veillonella parvula | 2 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 15 µg |
| b) | - Ribosomes of Escherichia coli | 3 µg |
| | - Ribosomes of Salmonella typhimurium | 3 µg |
| | - Ribosomes of Shigella dysenteriae | 3 µg |
| | - Ribosomes of Staphylococcus aureus | 3 µg |

- Membrane proteoglycans of Kelbsiella pneumoniae 18 µg

c) - Ribosomes of type A Candida albicans 3.5 µg
- Ribosomes of type B Candida albicans 3.5 µg
- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

d) - Ribosomes of group A Neisseria meningitidis 3.5 µg
- Ribosomes of group C Neisseria meningitidis 3.5 µg
- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

e) - Ribosomes of type a Haemophilus influenzae 3.5 µg
- Ribosomes of type b Haemophilus influenzae 3.5 µg
- Membrane proteoglycans of Klebsiella pneumoniae 15.0 µg

f) - Ribosomes of type 1 Streptococcus pneumoniae 3 µg
- Ribosomes of type 2 Streptococcus pneumoniae 3 µg
- Ribosomes of type 4 Streptococcus pneumoniae 3 µg
- Ribosomes of type 19 Streptococcus pneumoniae 3 µg
- Membrane proteoglycans of Klebsiella pneumoniae 20 µg.

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g) - Ribosomes of group L Streptococcus pyogenes 5 µg
- Ribosomes of group C Streptococcus pyogenes 5 µg

| | | |
|---|---|---|
| | - Ribosomes of Streptococcus suis | 5 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 10 µg |
| h) | - Ribosomes of Staphylococcus epidermidis | 1.5 µg |
| | - Ribosomes of type 7 Staphylococcus aureus | 1.5 µg |
| | - Ribosomes of Staphylococcus aureus | 1.5 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 7.5 µg |
| i) | - Ribosomes of Staphylococcus (Mérimée strain) | 3.5 µg |
| | - Ribosomes of Staphylococcus aureus | 3.5 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 15.0 µg |
| j) | - Ribosomes of Staphylococcus aureus | 5 µg |
| | - Ribosomes of Staphylococcus uberis | 5 µg |
| | - Ribosomes of Streptococcus dysgalactiae | 5 µg |
| | - Membrane proteoglycans of biotype a Klebsiella pneumoniae | 20 µg |